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Cell-cycle-dependent translation of histone mRNAs is the key control point for regulation of histone biosynthesis in *Leishmania infantum*

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The cell-cycle-dependent expression of the four core histones (H2A, H2B, H3 and H4) has been studied in the protozoan parasite *Leishmania infantum*. For that purpose, the cell cycle was arrested by incubation of promastigotes with the DNA synthesis inhibitor hydroxyurea, which induced an accumulation of cells stalled in G_1 phase. Hydroxyurea release resulted in a semi-synchronous entry into the cell cycle, as determined by flow cytometry. The steady-state levels of histone mRNAs in the G_1, S and G_2/M phases were found to be constant along the cell cycle. However, the levels of histone synthesis increased when parasites enter the S phase, in agreement with previous results showing that histone synthesis in *Leishmania* is tightly coupled with DNA replication. In addition, we analysed the distribution of histone mRNAs on polyribosomes at different stages of the cell cycle by separation of cytoplasmic RNAs in sucrose gradients. Remarkably, a drastic change in the polysome profiles of histone mRNAs was observed during the progression from G_1 to S phase. Thus, in the S phase, histone mRNAs are present in ribosome-bound fractions, but in the G1 phase, the histone transcripts are exclusively found in the ribosome-free fractions. These results support a regulatory model in which the cell-cycle-regulated synthesis of histones in *Leishmania* is controlled through a reversible interaction between translational repressors and histone mRNAs.

Key words: cell cycle, histone biosynthesis, *Leishmania*, polysomal profile, sucrose gradient, translational efficiency.

INTRODUCTION

Parasitic protozoa of the genus *Leishmania* are the etiological agents of a variety of diseases in human and other vertebrates known as leishmaniases. *Leishmania* and other related kinetoplastids are among the most primitive eukaryotes [1] and, perhaps as a reflection of their phylogenetic location, they exhibit many unusual features of gene organization and expression. Protein coding genes are organized in large clusters on the same DNA strand [2], which are transcribed from undefined promoters as large polycistronic precursor RNAs. Maturation of all pre-mRNAs involves two RNA processing reactions: *trans*-splicing [3] and 3′-end polyadenylation [4]. Gene regulation is one of the most intriguing aspects of trypanosomatid biology, since trypanosomes do not seem to use the process of transcription initiation as a regulatory step [5]. Changes in the steady-state levels of regulated transcripts in these parasites have been related with post-transcriptional events involving sequences present mainly in the 3′-untranslated regions [5,6]. Consequently, these protozoa constitute an excellent model to study post-transcriptional regulation in eukaryotes.

The genes encoding histones, the major proteinaceous constituents of the eukaryotic chromatin, have been extensively used as models for studies on gene expression in a variety of organisms. In most cells, the synthesis of histones is tightly coupled with the rate of DNA replication [7,8]. The synthesis of replication-dependent histones is restricted to the S phase of the cell cycle by mechanisms operating at the levels of histone transcription, pre-mRNA processing and mRNA stability. The combined effect is to regulate the production of histone mRNAs. For example, when mammalian cells progress from G_1 to S phase, the rate of histone gene transcription increases 3–5-fold, and the efficiency of histone pre-mRNA processing increases 8–10-fold, resulting in a 35-fold increase in histone levels [9]. Conversely, when cells exit the S phase, the half-life of histone mRNAs is drastically reduced, and the efficiency of processing and the rate of transcription are also reduced, resulting in a rapid decrease in histone mRNA levels [8].

In recent years, several studies focusing on histone gene expression in kinetoplastids have been reported. As seen in all organisms, the histone biosynthesis in trypanosomes is tightly coupled with DNA synthesis [10–12]. However, the regulatory mechanisms show differences from those operating in most eukaryotes, and peculiarities have been observed among different genera of the order *Kinetoplastida*. Common to all trypanosomatids is the fact that histone gene expression is controlled by post-transcriptional events rather than by changes in transcription rates. In *Trypanosoma cruzi*, inhibition of DNA synthesis results in the decrease in histone mRNAs [13–15]. Moreover, measurements of the steady-state levels of histone mRNAs along the *T. cruzi* cell cycle indicate that histone mRNAs accumulate in the S phase [16,17]. Similarly, the abundance of *H2B* mRNA in *T. brucei* decreased after culturing in the presence of HU (hydroxyurea), an inhibitor of DNA synthesis [18]. Furthermore, *in situ* hybridization studies showed that histone mRNAs are only detectable during the S phase in *T. brucei* [19]. Unlike the situation in most other eukaryotes, including *Trypanosoma*, the histone mRNA levels in *Leishmania* do not decrease after treatments with inhibitors of DNA synthesis [14,20,21]. Although the steady-state level of histone mRNAs remains unaffected, the synthesis of histones is tightly coupled with DNA replication in *Leishmania*, suggesting a regulatory mechanism operating at the translational level [12].

To obtain a more detailed knowledge of the mechanisms coupling histone gene expression with DNA replication in *Leishmania*, we have investigated here the translational status of histone mRNAs during the *Leishmania* cell cycle. The present study indicates that histone transcripts are bound to ribosomes during

Abbreviations used: Hsp70, heat-shock protein 70; HU, hydroxyurea; RNP, ribonucleoprotein.

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the S phase, but not during the G_1 phase, even though the histone mRNA levels remain constant along the cell cycle. These results support a model in which the regulation of histone expression in *Leishmania* is achieved by factors that block the translation of histone mRNAs when DNA synthesis is stopped.

EXPERIMENTAL

Parasites, HU treatment and metabolic labelling of DNA and proteins

Promastigotes of *Leishmania infantum* (M/CAN/ES/96/BCN150) were grown at 26 °C in RPMI 1640 medium (Gibco, Paisley, U.K.), supplemented with 10 % (v/v) heat-inactivated foetal calf serum (ICN Pharmaceuticals, Basingstoke, Hants, U.K.). Experimental cultures were initiated at 1×10^6 promastigotes/ml and harvested for study in the exponential phase of growth [$(5–8) \times 10^6$ promastigotes/ml].

For the synchronization of DNA replication, parasites in the exponential phase of growth (5×10^6 promastigotes/ml) were treated with 5 mM HU for 12 h. Afterwards, parasites were harvested, washed twice with PBS and resuspended in fresh medium without the drug. At different periods of time, 1 ml aliquots were removed from the cultures, pelleted and resuspended in 200 μl of the complete RPMI medium containing 20 μCi of [*methyl*-^{3}H]-thymidine (2.0 Ci/mmol; Amersham Biosciences, Little Chalfont, Bucks., U.K.). After incubation for 1 h at 26 °C, thymidine incorporation into DNA was determined using the MultiScreen Assay System (Millipore, Molsheim, France). For protein labelling, 10 ml aliquots were removed at different periods of time, and after washing twice with PBS, parasites were resuspended in 100 μl of Dulbecco's medium (without methionine) and 7 μl of the Pro-mix™ ^{35}S *in vitro* cell-labelling mix containing L-[^{35}S]methionine and L-[^{35}S]cysteine (1 mCi/ml and 1000 Ci/mmol respectively; Amersham Biosciences), and the culture was incubated for 60 min at 26 °C. The labelling medium was also supplemented with 5 mM HU for aliquots taken at zero time.

Flow-cytometric analysis

For flow-cytometric analysis, 4×10^6 promastigotes were harvested by centrifugation at 660 ***g***, washed twice with PBS, resuspended in 1 ml of fixative solution (30 % PBS/70 % methanol) and incubated at 4 °C for 1 h. Afterwards, parasites were collected by centrifugation, resuspended in PBS containing 20 μg/ml of RNase A (Roche, Mannheim, Germany) and incubated for 20 min at 37 °C. After incubation, the cells were harvested, resuspended in 1 ml of citrate buffer (45 mM $MgCl_2$/30 mM sodium citrate/20 mM Mops, pH 7.0/0.1 % Triton X-100), and stained by the addition of 50 μg of propidium iodide (Sigma, St. Louis, MO, U.S.A.) followed by incubation at 37 °C for 20 min. Afterwards, stained cells were washed twice with PBS containing 5 % foetal calf serum. The samples were stored at 4 °C in the dark until analysis. Fluorescence was determined by flow cytometry on an FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.).

RNA purification and Northern-blot analysis

Standard techniques of molecular biology were used [22]. *L. infantum* RNA was isolated as described previously [23]. Total RNA (5 μg/lane) was size-separated on 1 % (w/v) agarose–formaldehyde gels [24] and electrophoresed to nylon membranes using an LKB system (Amersham Biosciences). DNA probes were labelled by nick translation as described previously [22]. Hybridization was performed in 50 % (v/v) formamide, 6 × SSC buffer (0.9 M NaCl/0.09 M sodium citrate), 0.1 % SDS and 0.25 mg/ml of herring sperm DNA at 42 °C overnight. Final post-hybridization washes were performed in 0.1 × SSC (15 mM NaCl/1.5 mM sodium citrate)/0.2 % SDS at 50 °C for 1 h. For re-use, blots were treated with 0.1 % SDS for 15 min at 95 °C to remove the previously hybridized probes.

Analysis of polysomal distribution of histone mRNAs by sucrose gradients

Subcellular fractionation in linear 15–40 % (w/v) sucrose gradients was performed as described by Müllner and García-Sanz [25]. Briefly, *L. infantum* parasites (2.5×10^8 promastigotes) were harvested, washed twice with prechilled PBS and resuspended in 1 ml of lysis buffer (10 mM Tris/HCl, pH 8.0/150 mM NaCl/1.5 mM $MgCl_2$/0.5 % Nonidet P40), supplemented with 240 units of SUPERase-In (Ambion, Austin, TX, U.S.A.). Lysis was favoured by pipetting up and down ten times. After lysis, samples were microfuged at 3000 ***g*** for 2 min at 4 °C to pellet the nuclei. The supernatant was supplemented with 0.6 mg/ml heparin, 150 μg/ml cycloheximide, 20 mM dithiothreitol and 1 mM PMSF, and microfuged at 13 000 ***g*** for 5 min at 4 °C to remove mitochondria and membrane debris. The supernatant was layered on 11 ml sucrose gradient and centrifuged in an SW41T rotor at 170 000 ***g*** for 2 h at 4 °C. After completion, 15 fractions of 800 μl were collected from the top of the gradient. SDS [1 % (w/v) final concentration], EDTA (pH 8.0; final concentration, 10 mM) and proteinase K (final concentration, 200 mg/ml) were added to each fraction and incubated for 30 min at 37 °C. After extraction with a mixture of phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.), the RNA from the fractions was precipitated with ethanol at − 20 °C. RNA samples purified from each fraction were separated on denaturing 1 % agarose–formaldehyde gels and transferred on to nylon membranes.

Protein analyses

Nuclei from *L. infantum* promastigotes were isolated as described previously [12]. Nuclear preparations were analysed by SDS/PAGE (10–14 % linear gradient gels) at 10 mA for 12 h using the Hoefer Scientific Instrument protein system (Amersham Biosciences).

Nuclear and cytosolic fractions were separated by the method of Schreiber et al. [26]. Briefly, 2×10^7 promastigotes were pelleted, washed twice in ice-cold PBS, resuspended in 400 μl of prechilled buffer A (10 mM Hepes, pH 7.5/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM dithiothreitol/0.5 mM PMSF), and incubated for 15 min on ice. After incubation, Nonidet P40 was added to a final concentration of 0.6 %, and cells were lysed by vigorous vortex-mixing for 10 s, and immediately pelleted in a microfuge (13 000 ***g***). The supernatant (cytosolic fraction) was mixed 1:1 in 2 × Laemmli's buffer [27]. The pelleted nuclei were resuspended in 1 × Laemmli's buffer. Total cytosolic and nuclear proteins were separated by SDS/PAGE (13 % gels) in a Mini-protein system (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For immunoblot analysis, the electrophoresed proteins were transferred on to nitrocellulose membranes (Amersham Biosciences). The membrane was blocked with 5 % (w/v) non-fat dried milk powder in PBS and 0.5 % Tween 20. The filters were probed with anti-H2A sera obtained from a rabbit immunized with the recombinant *L. infantum* histone H2A [12]. An anti-rabbit IgG–peroxidase immunoconjugate (Nordic Immunologic, Tilburg, The Netherlands) was used as secondary antibody, and the specific

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binding was revealed with the ECL® Western-blot detection system (Amersham Biosciences).

Analysis of the *de novo* Hsp70 (heat-shock protein 70) synthesis along the cell cycle was performed on ^{35}S-labelled promastigotes by immunoprecipitation. After labelling (see above), parasites (6×10^7 cells) were harvested by centrifugation and incubated for 15 min on ice in 100 μl of lysis buffer, containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % (v/v) Triton X-100 and a protease inhibitor cocktail (1 mM PMSF, 8 μg/ml leupeptin, 4 μg/ml pepstatin and 4 μg/ml aprotinin). Lysates were sonicated for 15 min in a water bath for clearance of nucleic acids. The samples were centrifuged at 13 000 ***g*** for 15 min and the protein content of the supernatants was measured using the Bio-Rad protein assay reagent. The protein extract (150 μg) was mixed with 20 μl of rabbit antiserum against *L. infantum* Hsp70 [28] and incubated on an orbital rotator for 15 h at 4 °C. Agarose beads (15 μl), conjugated with Protein A (Sigma), were equilibrated in 50 μl of lysis buffer and added to the *Leishmania* extract/Hsp70 antiserum mixture. The mixture was incubated on a rotator for 2 h at 4 °C. The beads were collected by centrifugation and washed three times with 0.5 ml of buffer A (10 mM Tris/HCl, pH 8.0/30 mM NaCl/2 % Triton X-100), twice in 0.5 ml of buffer B (10 mM Tris/HCl, pH 8.0/50 mM NaCl/2 % Triton X-100) and once in 0.5 ml of buffer C (10 mM Tris/HCl, pH 8.0/0.05 % Triton X-100). Finally, the beads were resuspended in 60 μl of 2 × Laemmli's buffer. Immunoprecipitated proteins were resolved by SDS/PAGE (7.5 % gel), transferred on to a nitrocellulose membrane and exposed to an autoradiographic film. Total amount of immunoprecipitated Hsp70 was analysed by Western blotting using the anti-Hsp70 serum.

Quantitative analysis

The autoradiographs were scanned with a laser densitometer (Image Quant™ version 3.0; Molecular Dynamics). Measurements were performed under conditions in which a linear correlation existed between the amount of proteins or RNA and the band intensity on the autoradiographs.

RESULTS

Cell-cycle arrest of *L. infantum* promastigotes with 5 mM HU

Different phases of the *Leishmania* cell cycle were determined by flow-cytometric analysis of DNA content in promastigotes, stained with propidium iodide. Data obtained with exponentially growing cultures of *L. infantum* promastigotes showed the following percentages of cells in the different cell-cycle phases (Figure 1): 65 % in G_1 phase, 23 % in S phase and 12 % in G_2/M phase. In a previous work, we validated the use of 5 mM HU, an inhibitor of the ribonucleotide reductase, to achieve a reversible inhibition of DNA synthesis in *L. infantum* [12]. As shown in Table 1, treatment of promastigotes for 12 h with 5 mM HU induced a marked inhibition of DNA synthesis that was reversed after removing the drug. In fact, 1 h after HU removal, *Leishmania* promastigotes incorporated [*methyl*-^{3}H]thymidine actively, suggesting that DNA synthesis is re-started shortly after clearing the drug. Maximum DNA synthesis was observed 3 h after HU removal, when the level of [*methyl*-^{3}H]thymidine incorporation was 24-fold higher compared with the HU-treated cells and 5-fold higher than that of the untreated control cultures (Table 1). This was taken as an indication that HU was arresting *Leishmania* cell cycle at the G_1/S phase boundary. This suggestion was further supported by the results from flow cytometry (Figure 1). After 12 h of HU treatment, most of the cells were found to be arrested in the G_1 phase. After HU removal, the parasites proceeded through the cell cycle. Even 3 h after the release, most of the cells were in the S phase (approx. 63 %), a result that coincides with the results of [*methyl*-^{3}H]thymidine incorporation (Table 1). Promastigotes displayed, 9–11 h after the release, a cell-cycle distribution similar to that of asynchronously growing parasites (i.e. exponential-phase promastigotes; Figure 1). In

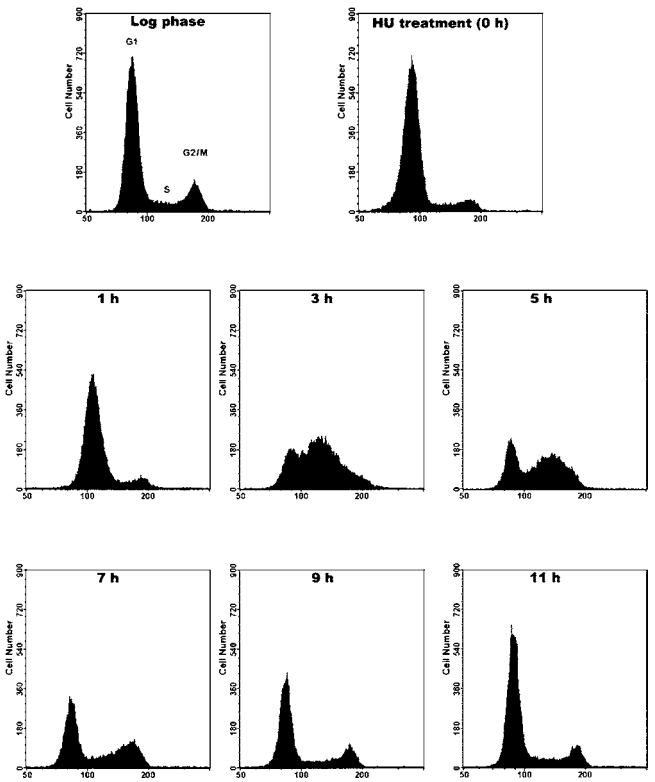

Figure 1 Analysis of the DNA content in *L. infantum* promastigote cells after release from HU-induced arrest

Cells were collected from cultures in the exponential phase of growth (Log phase = exponential phase), after 12 h of incubation with 5 mM HU (0 h), and at the indicated time periods after removing the drug. Cells were stained with propidium iodide (PI) and the DNA content per cell was analysed by flow cytometry. The graphs are histograms of relative PI fluorescence (proportional to DNA content) plotted against frequency of events per channel (counts), equivalent to cell number. Each histogram represents data collected from 20 000 events/sample. The position for G_1, S and G_2/M, and the subsequent statistical analyses, were derived from actual data using the Cell Quest™ (Becton Dickinson) software.

Table 1 [*methyl*-^{3}H]Thymidine incorporation into DNA after the removal of HU blockade

After 12 h of HU treatment and wash-out of the drug, incorporation of [*methyl*-^{3}H]thymidine into DNA was measured over a period of 11 h. Results are expressed as the means ± S.D. for three triplicates of one representative experiment. The [*methyl*-^{3}H]thymidine incorporation of a control untreated culture was 15 213 ± 1092 c.p.m.

Time (h)	[Methyl-^{3}H]thymidine incorporated (c.p.m.)
0	2928 ± 365
1	50 609 ± 2233
3	71 292 ± 6677
5	63 347 ± 2757
7	53 657 ± 5145
9	25 389 ± 1841
11	18 828 ± 2745

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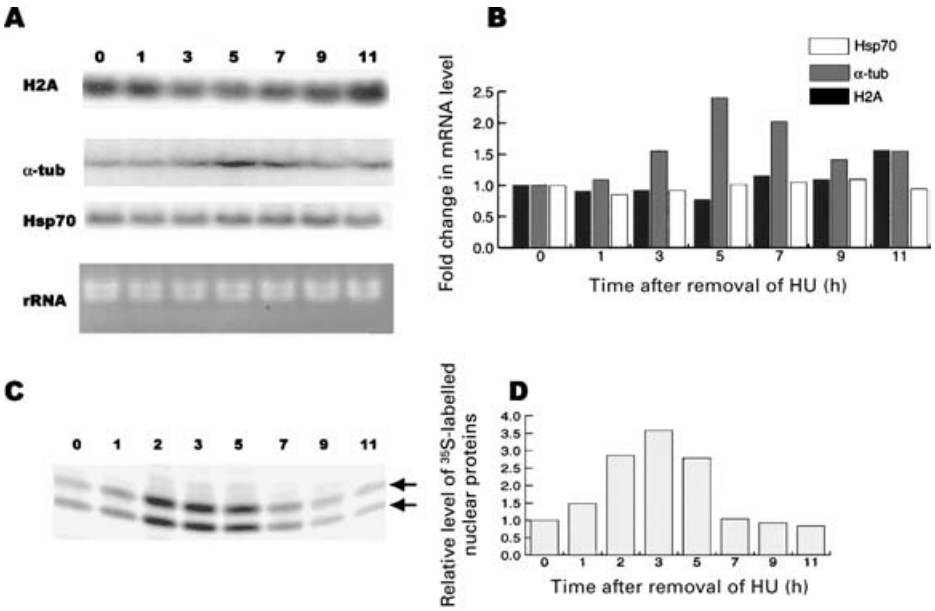

Figure 2 Analysis of the cell-cycle-dependent expression of *L. infantum* histones

(**A**) Northern-blot analysis of total RNA samples (5 μg) from promastigotes, treated with 5 mM HU either for 12 h (lane 0) or at the indicated times (in h) after removal of the drug. The blot was sequentially probed with cDNAs for *L. infantum* histone H2A (clone cL72) [37], *T. cruzi* α-tubulin (clone pTcα3) [38] and *L. infantum* Hsp70 (clone B2) [39]. Ethidium bromide staining of the corresponding gel is also shown (panel rRNA). (**B**) Histogram corresponding to the densitometric analysis of Northern blots, showing the fold change in *H2A*, *Hsp70* and *α-tubulin* mRNA levels (relative to the 0 h time point) after HU release. (**C**) Autoradiographic exposure of a SDS/10–14 % linear gradient polyacrylamide gel from ^{35}S-labelled nuclear proteins (20 μg) prepared from *L. infantum* promastigotes treated with 5 mM HU for 12 h (lane 0) or at the indicated time periods (in h) after removal of the drug. (**D**) Histogram showing the densitometric quantification of the autoradiograph shown in (**C**). Values were normalized relative to the value at time 0 h (taken arbitrarily as 1).

fact, subsequent waves of synchrony were not observed in these parasites, and the populations could not be distinguished from asynchronous cultures (results not shown). In conclusion, both [*methyl*-^{3}H]thymidine incorporation and flow-cytometry data indicated that HU promotes an arrest of the *Leishmania* growth at the G_1/S boundary and that the mid-S phase is reached 3 h after the removal of the DNA synthesis inhibitor.

Cell-cycle expression of *Leishmania* histones

In a previous work, we have shown that in *Leishmania* there is a close relationship between DNA replication and histone synthesis [12]. However, inhibition of DNA synthesis did not affect either the transcriptional activity of histone genes or the steady-state levels of histone mRNAs. Therefore it was concluded that histone synthesis in *Leishmania* is linked to DNA replication by a translational control [12]. To determine how the *Leishmania* histone gene expression varies along the cell cycle, a Northern blot of RNA isolated from parasites at various time points, after the release from HU blockade, was hybridized with an H2A probe (Figure 2A, panel H2A). As loading control, the same blot was also hybridized with an α-tubulin probe (Figure 2A, panel α-tub) and an Hsp70 probe (Figure 2A, panel Hsp70). After each hybridization, the radioactive signals were quantified (Figure 2B). The level of the *H2A* mRNAs was found to remain constant throughout the cell cycle. Similar results were observed when the blot was sequentially probed with the *H2B*, *H3* and *H4* genes (results not shown), indicating that the mRNA steady-state levels for the four core histones in *Leishmania* are not cell-cycle-regulated. The abundance of the Hsp70 mRNAs was also found to remain constant along the cell cycle, whereas a 2.4-fold increase in the α-tubulin mRNA levels was detected at the late S phase of the cell cycle (Figure 2B).

Next, we analysed the *de novo* synthesis of *Leishmania* histones at different times after HU removal. Similar protein amounts from nuclear extracts prepared from cells at various time periods after the release from HU blockade were resolved on 10–14 % linear gradient SDS/polyacrylamide gels. Figure 2(C) shows the autoradiograph of the gel in the region where two prominent bands, corresponding to the core histones, are found. Densitometric measurements (Figure 2D) indicated that ^{35}S-labelling of histones significantly increased when cells enter the S phase. Interestingly, the labelling of histones decreased to initial levels (parasites arrested at G1 phase, lane 0 in Figure 2C) at 7 h after HU removal, when parasites proceeded towards G_2/M phase (Figure 1), suggesting that histone synthesis is restricted to the S phase of the *Leishmania* cell cycle. Alternatively, it could be hypothesized that the histone synthesis remains constant along the cell cycle but the newly synthesized histones are stored in the cytoplasm until DNA replication takes place. To rule out a differential nucleo-cytoplasmic transport of the histones, cytoplasmic and nuclear fractions were prepared from *L. infantum* promastigotes at different times after HU removal (Figure 3). After immunoblotting analysis using an antibody raised against *Leishmania* histone H2A, it became clear that this histone is not detected in the cytoplasm at any time along the *Leishmania* cell cycle. Therefore the possibility that histones are stored in the *Leishmania* cytoplasm in a significant amount can be excluded. It may be noted that if histones were stored in the cytoplasm at the levels required for assuring chromatin duplication, the amount of histones would be large and, therefore, they should be detected in the cytoplasm by Western blotting. Altogether, our results indicate that histone biosynthesis in *Leishmania* promastigotes is restricted to the S phase through a translational control on the histone mRNAs.

The polysomal profile of histone mRNAs varies along *Leishmania* cell cycle

Since there are large changes in histone synthesis as promastigotes enter the S phase (Figures 2C and 2D) without changes in histone

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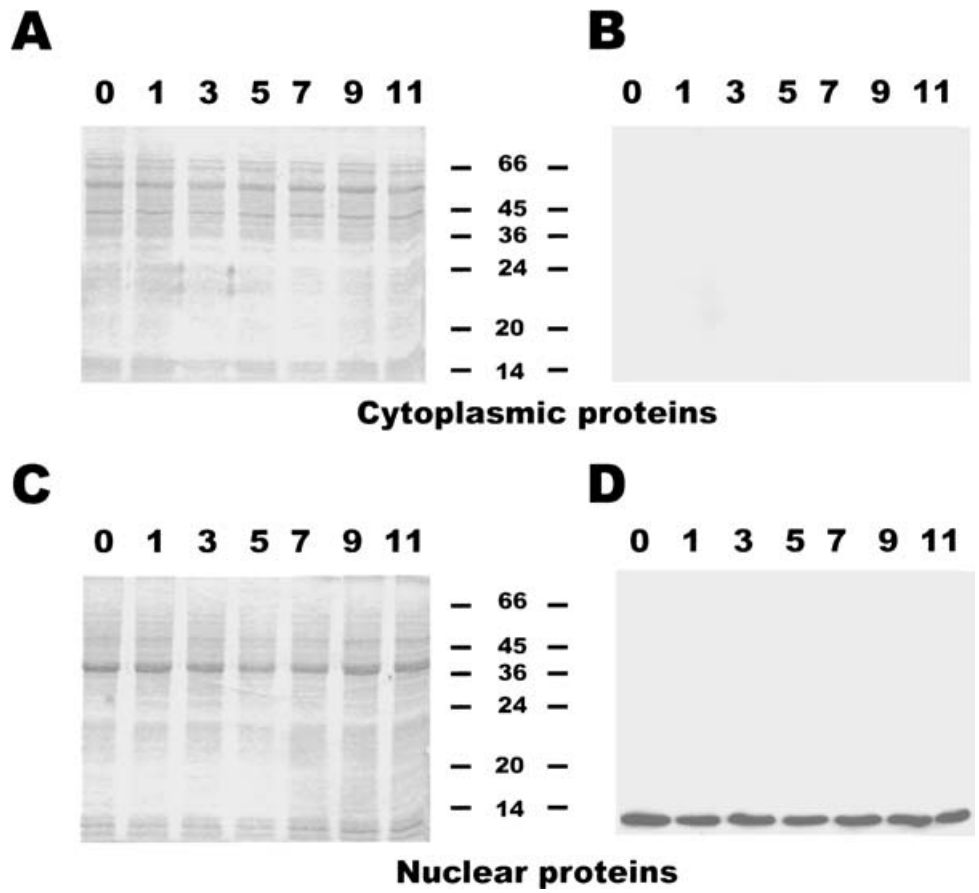

Figure 3 Histones are not stored in the cytoplasm

At the indicated time periods (in h) after HU release, *Leishmania* promastigotes were harvested for cellular fractionation. Samples (5 μg) of cytoplasmic (**A**, **B**) or nuclear proteins (**C**, **D**) were resolved on SDS/13 % polyacrylamide minigels; pairs of gels were either stained with Coomassie Blue (**A**, **C**) or blotted on to nitrocellulose filters (**B**, **D**). The blots were incubated with an anti-H2A antibody purified by affinity chromatography from a polyclonal serum obtained from a rabbit immunized with the recombinant *L. infantum* histone H2A. Molecular-mass sizes are indicated in kDa.

mRNA levels (Figures 2A and 2B), it is plausible that histone expression could be regulated through differences in the translational accessibility of histone mRNAs in the different phases of the *Leishmania* cell cycle. Following the method of Müllner and García-Sanz [25], we analysed the distribution of histone mRNAs on polyribosomes during the progression from G_1 to S phase. The translational efficiency of a given mRNA species is characterized by its ribosome loading profile, i.e. the distribution between ribosome-free mRNAs and polysome-bound mRNAs [29]. Figure 4(A) shows the ribosomal distribution of *L. infantum* promastigotes in a linear 15–40 % sucrose gradient as deduced from the ethidium bromide staining of RNAs present in each fraction separated on a 1 % agarose–formaldehyde electrophoresis gel. It must be noted that the ribosomes of *Leishmania* are atypical in rRNA composition of the large subunit, which contains two large (24Sα and 24Sβ) and six small rRNA molecules, instead of the typical 28 S rRNA of most eukaryotes [30]. Based on the distribution of rRNA, fractions 1–4 must be considered to be free of functional ribosomes, since either they do not contain rRNAs or the rRNAs are not in equimolecular amounts. Once the ribosomal profile of *L. infantum* promastigotes was established, we analysed the distribution of histone mRNAs in two cell-cycle phases: G_1 phase (parasites treated for 12 h with 5 mM HU) and mid-S phase (3 h after the removal of the drug). The distribution along the sucrose-gradient fractions of the four core histone mRNAs is shown in the Northern blots of Figure 4(B). All the histone transcripts concentrated proximal to the top of the gradient (fractions 1–3) during the G_1 phase, suggesting that they are not bound to functional ribosomes in this phase. In contrast, all the histone transcripts shifted to polyribosomal fractions during the S phase, indicating that they are being translated actively. Noticeably, the polysome profiles of histones H2B and H3 mRNAs (each one encoded by two different-sized mRNAs) were found to be similar for both transcripts. The slight differences existing in the ribosome loading profiles of the four histone mRNAs are probably due to experimental variations of the sucrose gradients (it must be noted that each blot corresponds to an independent experiment).

The polysome profiles of two non-histone genes were examined: α-*tubulin* (Figure 4C) and *Hsp70* (Figure 5). The polysome profile of the α-*tubulin* mRNA indicated that this transcript is in an active translational status during G_1 and S phases of the *Leishmania* cell cycle. The distribution of the *Hsp70* mRNAs on polyribosomes during the progression from G_1 to S phase indicated that the *Hsp70* mRNAs are also in an active translational status in both phases of the *Leishmania* cell cycle, although in the S phase a higher ribosome loading was detected (Figure 5A). In parallel, the rate of the *de novo* synthesis of Hsp70 was determined by immunoprecipitation with an anti-Hsp70 antiserum on protein extracts from promastigote labelled with [^{35}S]methionine at various time periods after the release of HU (Figure 5B).

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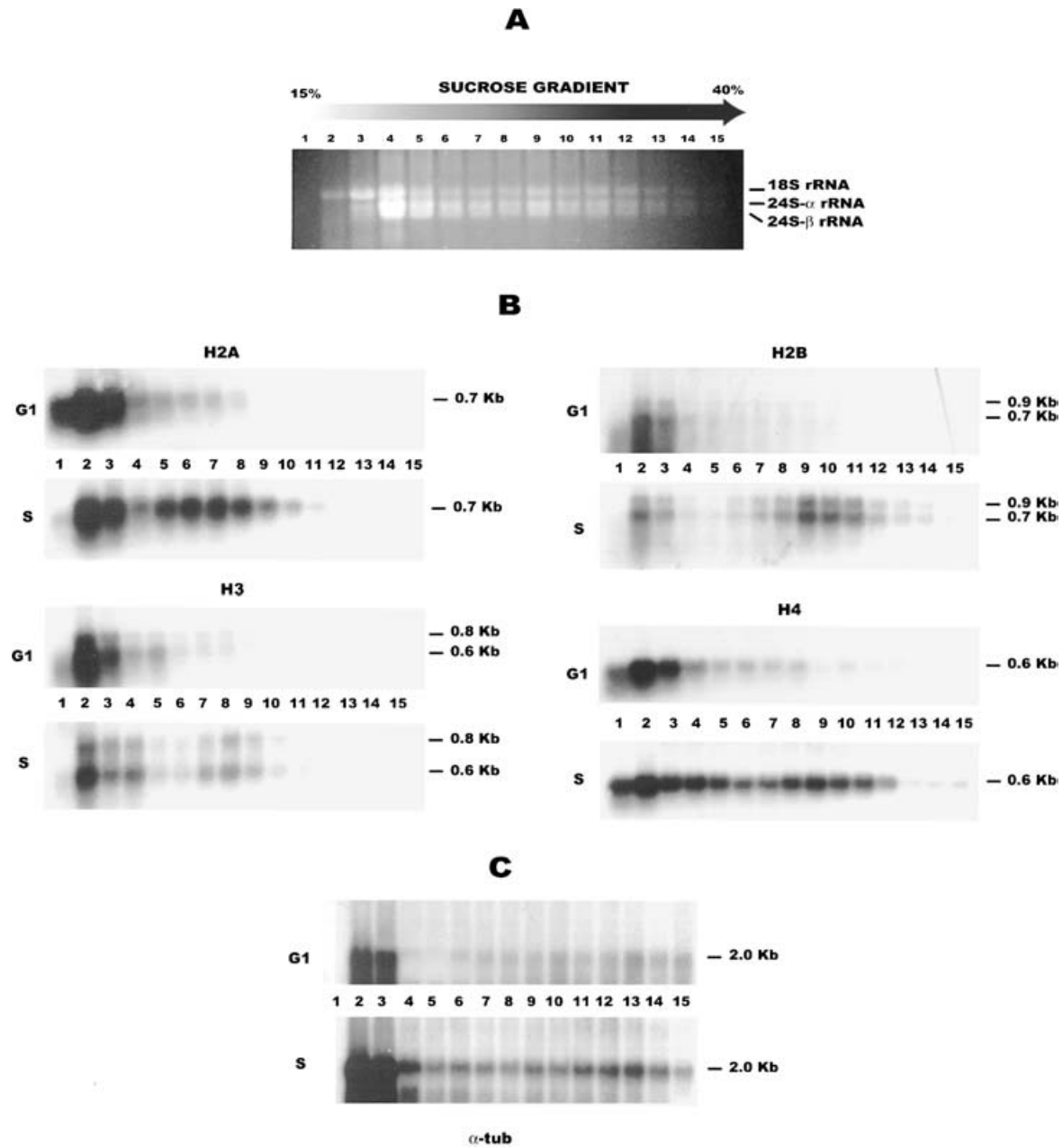

Figure 4 Analysis of RNA fractionation in sucrose gradients

(**A**) Cytoplasmic extracts from *L. infantum* promastigotes in exponential growth were layered on top of a 15–40 % linear sucrose gradient. After centrifugation, 15 fractions were collected from the sample; RNA was purified from each of them and resolved in a 1 % agarose–formaldehyde gel. The ethidium bromide staining of the gel is shown. The migrations of 18 S, 24 S-α and 24 S-β rRNAs are indicated. Fraction 1 corresponds to the top and fraction 15 to the bottom. (**B**) Sucrose gradients were prepared from parasites incubated with HU for 12 h (G_1 phase) or 3 h after the removal of the drug (S phase). After fractionation, RNA was isolated from each fraction, separated by electrophoresis and transferred on to nylon membranes. Blots were hybridized with the following probes: histone H2A (clone cL72 [37]), histone H2B [40], histone H3 (clone LiB6; [41]), and histone H4 (clone LiH4-1; [21]). (**C**) Northern blots prepared with RNA samples separated by sucrose gradients from promastigotes treated with HU for 12 h (G1 phase) or 3 h after removal of the drug (S phase) were hybridized with a *T. cruzi* α-tubulin probe [38]. Sizes are indicated in kb.

Analysis of the amount of radioactivity bound to the immunoprecipitated Hsp70 showed that this protein is actively translated during all the cell-cycle phases (Figure 5C), with a slight increase (1.6-fold) in the S phase.

Densitometric analysis of all the Northern blots are summarized in Figure 6. From this analysis, it became evident that all four histone mRNAs have very similar ribosome loading profiles and vary in the same fashion during the progression from G_1 to S phase. Thus, in the G_1 phase, all the histone mRNAs concentrate in ribosome-free fractions, whereas in the S phase, the histone mRNAs are distributed along the gradient, with a secondary peak in the ribosome-bound fractions. The percentages of histone mRNAs present in these secondary peaks were 37.91 % for H2A, 61.69 % for H2B, 40.74 % for H3 and 36.94 % for H4. In contrast, no significant variations were observed in the distribution of both *α-tubulin* and *Hsp70* mRNAs between the G_1 and S phases. A slight increase in the amount of ribosome-loaded *Hsp70* mRNAs in the S phase, relative to that present in the G_1 phase, was

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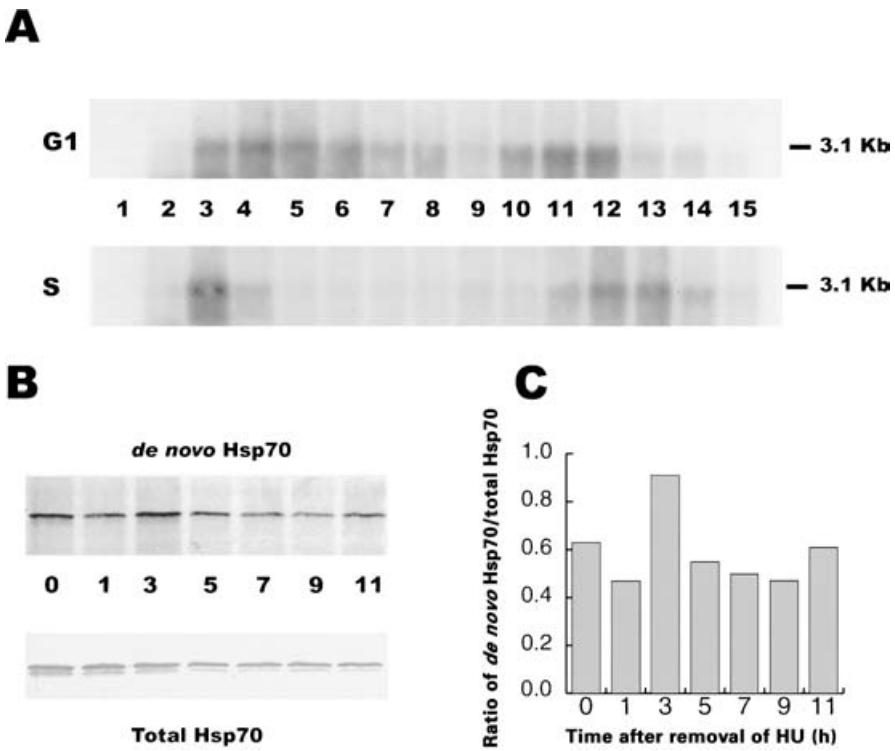

Figure 5 Expression of the Hsp70 along the cell cycle of *L. infantum*

(**A**) Northern blots prepared with RNA samples separated by sucrose gradients from promastigotes treated with HU for 12 h (G_1 phase) or 3 h after removal of the drug (S phase). Blots were hybridized with an Hsp70 probe (B2 cDNA) [39]. Sizes are indicated in kb. (**B**) Protein extracts from ^{35}S-labelled *L. infantum* promastigotes, treated with 5 mM HU either for 12 h (lane 0) or at the indicated time periods (in h) after removal of the drug, were used to immunoprecipitate the Hsp70 with a specific polyclonal antiserum. After immunoprecipitation, protein samples were separated by SDS/PAGE and transferred on to a nitrocellulose membrane. After autoradiographic exposure (panel *de novo* Hsp70), the membrane was incubated with the anti-Hsp70 serum to reveal the total amount of Hsp70 present in the samples (panel Total Hsp70). (**C**) Histogram showing the ratio between the *de novo* Hsp70 synthesis and total amount of Hsp70 as determined by densitometric quantification. The experiment was repeated twice and the mean values are represented.

observed, suggesting a higher translational efficiency of Hsp70 transcripts, which is in agreement with the 1.6-fold increase in *de novo* synthesis at this phase (Figure 5C). Altogether, these results suggest that the change in histone mRNA loading profiles is not a consequence of a global cellular process induced during the G_1 to S progression.

DISCUSSION

There is a selective pressure during evolution to confine the bulk of core histone biosynthesis to the S phase. The different organisms have evolved different mechanisms to restrict the synthesis of histones to the time of DNA replication. The major pathways contributing to this regulation operate at the levels of transcription, pre-mRNA processing and mRNA stability, and their combined effect is the control of the levels of histone mRNAs [7,8]. The results presented in this study, together with others from previous studies [12], demonstrate that the regulation of histone synthesis in *Leishmania* occurs exclusively at the translational level. To our knowledge, this is the first report of an organism in which the levels of histone mRNAs remain constant along the cell cycle. In a way, this finding may be considered as a new curiosity of these parasitic protozoa to be added to the list of surprising molecular mechanisms displayed by the *Kinetoplastida* [31].

The changes in the ribosome loading profiles of histone mRNAs during the cell cycle of *Leishmania* are a direct demonstration that the regulation of histone biosynthesis is occurring at the translational level. It has been established that the translational efficiency of a given mRNA species is characterized by its ribosome loading profile (i.e. its distribution between ribosome-free and polysome-bound fractions) [29]. The analysis of polysomal distribution of *Leishmania* histone mRNAs has shown that histone mRNAs are exclusively located in the ribosome-free fractions during the G_1 phase, indicating that they are in an impeded conformation for binding to ribosomes and, therefore, they cannot be translated. In contrast, in the S phase when an active synthesis of histones is detected, a large proportion of the histone mRNAs is bound to ribosomes. The molecular mechanisms underlying these changes in polysomal distribution of histone mRNAs in *Leishmania* are unknown, but some clues about the nature of the process can be deduced. On the basis of the fact that the histone mRNAs are exclusively found in the ribosome-free fractions in the G_1 phase and that these mRNAs seem to be resistant to degradation, it is probable that in the absence of DNA replication these transcripts are stored as cytoplasmic messenger RNPs (ribonucleoproteins) in a translation-inactive state. When cells enter S phase, putative translational repressors would leave the histone mRNAs and, then, they have access to the translational machinery. Therefore the expression of histone in *Leishmania* would be controlled by a mechanism operating at the level of translation initiation. To our knowledge, there are two examples in which the storage of histone mRNAs as RNPs leads to a translation-inactive form of the transcripts. In the primitive myxomycete *Physarum polycephalum*, it has been reported that both transcription of the histone genes and accumulation of histone mRNA occur at the G_2

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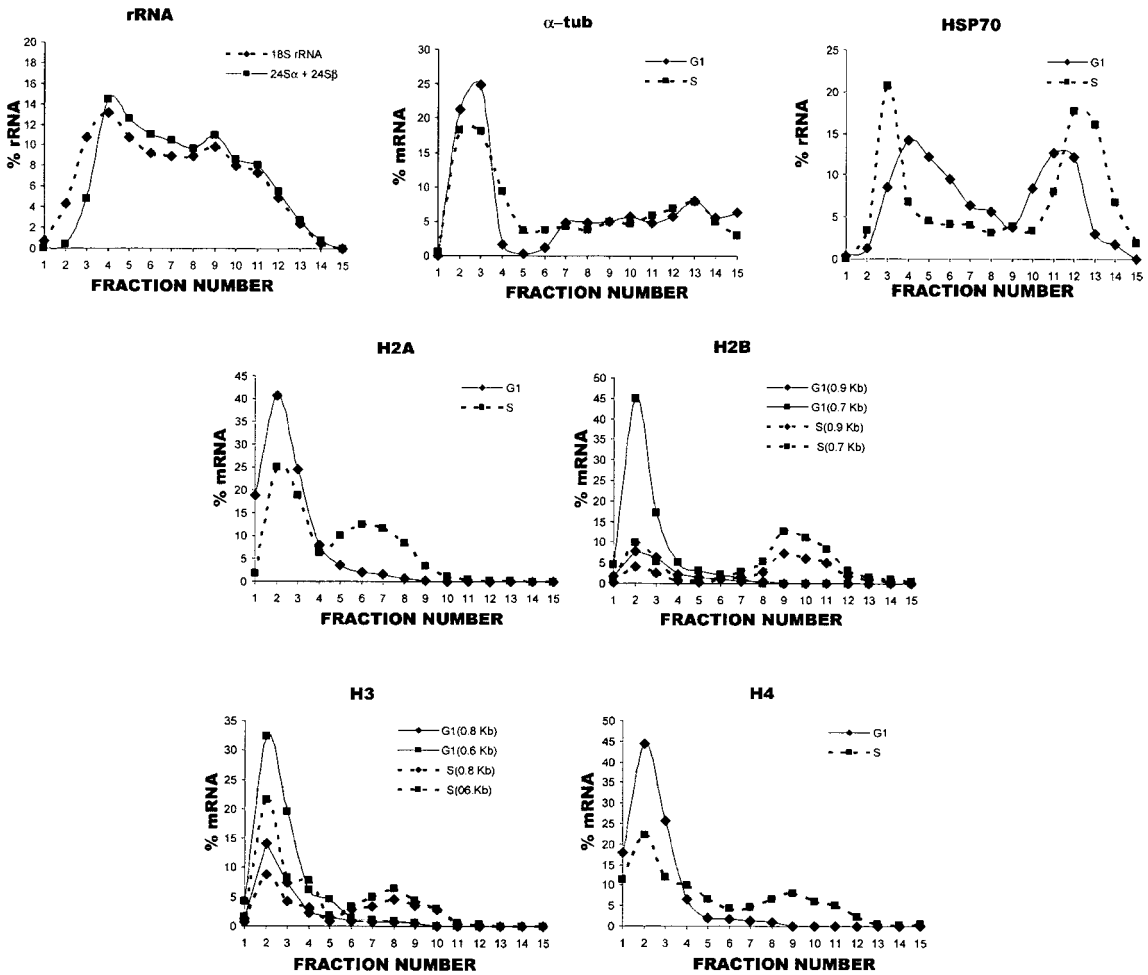

Figure 6 Densitometric measurements of the distribution of RNAs along the sucrose gradients

Densitometric analysis of the ethidium bromide-stained gel shown in Figure 4(A) (rRNA) and autoradiographs shown in Figure 4(B) (H2A, H2B, H3 and H4), Figure 4(C) (α-tub) and Figure 5(A) (Hsp70). Results are plotted as percentages of the total signal to allow direct comparison between the profiles of genes that can be expressed at different steady-state levels.

phase, in the absence of DNA synthesis. The histone mRNAs are stored in the cytoplasm as translationally inactive RNPs until the beginning of the next S phase [32,33]. Nevertheless, the translational control is a part of the multi-level regulation of histone gene expression that this organism possesses, whereas in *Leishmania* the control of histone expression occurs only at the translational level. The second example is found in *Xenopus* oocytes, in which the maternal histone mRNAs are stored as inactive RNPs and released for translation during the early stages of embryogenesis. In this case, specific proteins interacting with conserved stem-loop structures of histone mRNAs have been involved in the mechanism of activation–deactivation of histone mRNA translation [34]. This is a general mechanism, since many maternal mRNAs are synthesized during oogenesis and stored in the oocyte in a non-translated state until they are activated at oocyte maturation, fertilization or early embryonic development [35,36]. However, contrary to the mechanism controlling the histone expression in *Leishmania* that serves to restrict histone synthesis to S phase, in oocytes, the storage of histone mRNAs has been developed as a way to uncouple histone mRNA accumulation and DNA replication.

Taken together, the results presented in this study demonstrate that histone biosynthesis in *Leishmania* is regulated by a mechanism involving a translational repression that is exerted on histone mRNAs in the absence of DNA synthesis. To test this model, it would now be of great interest to identify the proteins which interact specifically with the histone mRNAs and to understand how they contribute to the regulation of the translation. Also, it will be crucial in the future to locate the *cis*-elements in the histone mRNAs that could be recognized by putative translational repressors.

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REFERENCES

1 Sogin, M. L., Elwood, H. J. and Gunderson, J. H. (1986) Evolutionary diversity of eukaryotic small-subunit rRNA genes. Proc. Natl. Acad. Sci. U.S.A. **83**, 1383–1387
2 Myler, P. J. and Stuart, K. D. (2000) Recent developments from the *Leishmania* genome project. Curr. Opin. Microbiol. **3**, 412–416
3 Agabian, N. (1990) *Trans* splicing of nuclear pre-mRNAs. Cell (Cambridge, Mass.) **61**, 1157–1160
4 LeBowitz, J. H., Smith, H. Q., Rusche, L. and Beverley, S. M. (1993) Coupling of poly(A) site selection and *trans*-splicing in *Leishmania*. Genes Dev. **7**, 996–1007
5 Clayton, C. E. (2002) Life without transcriptional control? From fly to man and back again. EMBO J. **21**, 1881–1888

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6 Stiles, J. K., Hicock, P. I., Shah, P. H. and Meade, J. C. (1999) Genomic organization, transcription, splicing and gene regulation in *Leishmania*. Ann. Trop. Med. Parasitol. **93**, 781–807
7 Osley, M. A. (1991) The regulation of histone synthesis in the cell cycle. Annu. Rev. Biochem. **60**, 827–861
8 Marzluff, W. F. and Duronio, R. J. (2002) Histone mRNA expression: multiple levels of cell cycle regulation and important developmental consequences. Curr. Opin. Cell Biol. **14**, 692–699
9 Harris, M. E., Bohni, R., Schneiderman, M. H., Ramamurthy, L., Schümperli, D. and Marzluff, W. F. (1991) Regulation of histone mRNA in the unperturbed cell cycle: evidence suggesting control at two posttranscriptional steps. Mol. Cell. Biol. **11**, 2416–2424
10 Sabaj, V., Diaz, J., Toro, G. C. and Galanti, N. (1997) Histone synthesis in *Trypanosoma cruzi*. Exp. Cell Res. **236**, 446–452
11 Marañón, C., Thomas, M. C., Puerta, C., Alonso, C. and López, M. C. (2000) The stability and maturation of the H2A histone mRNAs from *Trypanosoma cruzi* are implicated in their post-transcriptional regulation. Biochim. Biophys. Acta **1490**, 1–10
12 Soto, M., Quijada, L., Alonso, C. and Requena, J. M. (2000) Histone synthesis in *Leishmania infantum* is tightly linked to DNA replication by a translational control. Biochem. J. **346**, 99–105
13 Garcia-Salcedo, J. A., Oliver, J. L., Stock, R. P. and Gonzalez, A. (1994) Molecular characterization and transcription of the histone H2B gene from the protozoan parasite *Trypanosoma cruzi*. Mol. Microbiol. **13**, 1033–1043
14 Soto, M., Requena, J. M., Quijada, L. and Alonso, C. (1996) Organization, transcription and regulation of the *Leishmania infantum* histone H3 genes. Biochem. J. **318**, 813–819
15 Marañón, C., Puerta, C., Alonso, C. and López, M. C. (1998) Control mechanisms of the H2A genes expression in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **92**, 313–324
16 Recinos, R. F., Kirchhoff, L. V. and Donelson, J. E. (2001) Cell cycle expression of histone genes in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. **113**, 215–222
17 Sabaj, V., Aslund, L., Pettersson, U. and Galanti, N. (2001) Histone genes expression during the cell cycle in *Trypanosoma cruzi*. J. Cell. Biochem. **80**, 617–624
18 Garcia-Salcedo, J. A., Gijon, P. and Pays, E. (1999) Regulated transcription of the histone H2B genes of *Trypanosoma brucei*. Eur. J. Biochem. **264**, 717–723
19 Ersfeld, K., Docherty, R., Alsford, S. and Gull, K. (1996) A fluorescence *in situ* hybridisation study of the regulation of histone mRNA levels during the cell cycle of *Trypanosoma brucei*. Mol. Biochem. Parasitol. **81**, 201–209
20 Genske, J. E., Cairns, B. R., Stack, S. P. and Landfear, S. M. (1991) Structure and regulation of histone H2B mRNAs from *Leishmania enriettii*. Mol. Cell. Biol. **11**, 240–249
21 Soto, M., Quijada, L., Alonso, C. and Requena, J. M. (1997) Molecular cloning and analysis of expression of the *Leishmania infantum* histone H4 genes. Mol. Biochem. Parasitol. **90**, 439–447
22 Sambrook, L., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Plainview, NY
23 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. **162**, 156–159
24 Lehrach, H., Diamond, D., Wozney, J. M. and Boedtker, H. (1977) RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry **16**, 4743–4751
25 Müllner, E. W. and García-Sanz, J. A. (1997) Polysome gradients. In Manual of Immunology Methods, vol. 1 (Lefkovits, I., ed.), pp. 457–462, Academic Press, London
26 Schreiber, E., Matthias, P., Muller, M. M. and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. Nucleic Acids Res. **17**, 6419
27 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685
28 Rico, A. I., Angel, S. O., Alonso, C. and Requena, J. M. (1999) Immunostimulatory properties of the *Leishmania infantum* heat shock proteins HSP70 and HSP83. Mol. Immunol. **36**, 1131–1139
29 Pradet-Balade, B., Boulme, F., Mullner, E. W. and Garcia-Sanz, J. A. (2001) Reliability of mRNA profiling: verification for samples with different complexities. Biotechniques **30**, 1352–1357
30 Requena, J. M., Soto, M., Quijada, L., Carrillo, G. and Alonso, C. (1997) A region containing repeated elements is associated with transcriptional termination of *Leishmania infantum* ribosomal RNA genes. Mol. Biochem. Parasitol. **84**, 101–110
31 Donelson, J. E., Gardner, M. J. and El-Sayed, N. M. (1999) More surprises from *Kinetoplastida*. Proc. Natl. Acad. Sci. U.S.A. **96**, 2579–2581
32 Laffler, T. G. and Carrino, J. (1987) Cell-cycle-regulated translation of histone mRNA in *Physarum* plasmodia. J. Bacteriol. **169**, 2291–2293
33 Wilhelm, M. L., Toublan, B., Fujita, R. A. and Wilhelm, F. X. (1988) Histone H4 mRNA is stored as a small cytoplasmic RNP during the G2 phase in *Physarum polycephalum*. Biochem. Biophys. Res. Commun. **153**, 162–171
34 Wang, Z. F., Ingledue, T. C., Dominski, Z., Sanchez, R. and Marzluff, W. F. (1999) Two *Xenopus* proteins that bind the 3′ end of histone mRNA: implications for translational control of histone synthesis during oogenesis. Mol. Cell. Biol. **19**, 835–845
35 Hake, L. E. and Richter, J. D. (1997) Translational regulation of maternal mRNA. Biochim. Biophys. Acta **1332**, M31–M38
36 Dean, K. A., Aggarwal, A. K. and Wharton, R. P. (2002) Translational repressors in *Drosophila*. Trends Genet. **18**, 572–577
37 Soto, M., Requena, J. M., Gomez, L. C., Navarrete, I. and Alonso, C. (1992) Molecular characterization of a *Leishmania donovani infantum* antigen identified as histone H2A. Eur. J. Biochem. **205**, 211–216
38 Soares, C. M. A., de Carvalho, E. F., Urmenyi, T. P., Carvalho, J. F. O., de Castro, F. T. and Rondinelli, E. (1989) α- and β-tubulin mRNAs of *Trypanosoma cruzi* originate from a single multicistronic transcript. FEBS Lett. **250**, 497–502
39 Quijada, L., Soto, M., Alonso, C. and Requena, J. M. (1997) Analysis of post-transcriptional regulation operating on transcription products of the tandemly linked *Leishmania infantum* hsp70 genes. J. Biol. Chem. **272**, 4493–4499
40 Soto, M., Requena, J. M., Quijada, L., Perez, M. J., Nieto, C. G., Guzman, F., Patarroyo, M. E. and Alonso, C. (1999) Antigenicity of the *Leishmania infantum* histones H2B and H4 during canine viscerocutaneous leishmaniasis. Clin. Exp. Immunol. **115**, 342–349
41 Soto, M., Requena, J. M., Morales, G. and Alonso, C. (1994) The *Leishmania infantum* histone H3 possesses an extremely divergent N-terminal domain. Biochim. Biophys. Acta **1219**, 533–535

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